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Characterization of *Aplysia* Attractin, the First Water-borne Peptide Pheromone in Invertebrates

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Abstract. Although animals in the genus *Aplysia* are solitary during most of the year, they form breeding aggregations during the reproductive season. The aggregations contain both mating and egg-laying animals and are associated with masses of egg cordons. The egg cordons are a source of pheromones that establish and maintain the aggregation, but none of the pheromonal factors have been chemically characterized. In these studies, specimens of *Aplysia* were induced to lay eggs, the egg cordons collected and eluted, and the eluates fractionated by C18 reversed-phase HPLC. Four peak fractions were bioassayed in a T-maze. All four increased the number of animals attracted to a nonlaying conspecific and were thus subjected to compositional and microsequence analysis. Each contained the same NH_2-terminal peptide sequence. The full-length peptide ("attractin") was isolated from the albumen gland, a large exocrine organ that packages the eggs into a cordon. The complete 58-residue sequence was obtained, and it matched that predicted by an albumen gland cDNA. Mass spectrometry showed that attractin is 21 wt. % carbohydrate as the result of *N*-linked glycosylation. T-maze bioassays confirmed that the full-length peptide is attractive. Attractin is the first water-borne peptide pheromone characterized in molluscs, and the first in invertebrates.

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Abbreviations: ASW, artificial seawater; DHB, 2,5-dihydrobenzoic acid; Endo Glu-C, endoproteinase Glu-C; HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PCR, polymerase chain reaction; RP, reversed-phase.

Introduction

Pheromones play an important role in coordinating male and female reproductive behavior in many aquatic species, but relatively little is known about the identity of the water-borne factors or their specific activities. This is particularly true of water-borne peptide pheromones, which are difficult to isolate and characterize. The difficulties arise from the biological and chemical characteristics of the pheromonal system: (1) the small number of organisms that secrete the pheromone at any time; (2) the specific, but usually unknown, stimulus that induces pheromone secretion; (3) the relatively small amount of pheromone secreted; (4) the large volume of water into which secretion occurs—water that has contacted a variety of organisms and their products; and (5) the strong possibility that the pheromone may be rapidly degraded (Shimuzu, 1985).

Information generated over the last 30 years about reproductive activity in the marine opisthobranch mollusc *Aplysia* has lessened our concern about some of these problems—at least in this system. Field studies (Kupfermann and Carew, 1974; Audesirk, 1979; Susswein *et al.*, 1983, 1984) have shown that *Aplysia* is a solitary animal that moves into breeding aggregations during the summer reproductive season. The aggregations typically contain both mating and egg-laying animals and are associated with masses of recently deposited egg cordons. Most of the egg-laying animals simultaneously mate as females even though mating does not cause reflex ovulation (Blankenship *et al.*, 1983), suggesting that egg laying precedes

mating in the aggregation and that egg laying may release pheromones that establish and maintain the aggregation.

Similar observations have been made in the laboratory when animals were not individually caged (Audesirk, 1979; Blankenship *et al.*, 1983; Susswein *et al.*, 1983, 1984), and behavioral studies have shown that egg-laying animals with cordons are more attractive than sexually mature but nonlaying conspecifics (Aspey and Blankenship, 1976; Jahan-Parwar, 1976; Audesirk, 1977; Painter *et al.*, 1989). T-maze experiments demonstrate that at least some of the attractants derive from the egg cordon and are water-borne: (1) recent egg layers without egg cordons are no more attractive than nonlaying conspecifics; (2) recently deposited egg cordons are attractive, with or without the egg-laying animal, whereas sham cordons are not; and (3) both recently deposited egg cordons and their eluates increase the attractiveness of nonlaying conspecifics when placed in the adjacent seawater (Painter *et al.*, 1991; Painter, 1992). There are also contact pheromones on the egg cordon that can induce reproductive activity (Begnoche *et al.*, 1996).

Two potential tissue sources of the "cordon-derived" pheromonal attractants have been identified in T-maze experiments (Painter, 1992, 1993). The first is the albumen gland (Fig. 1), a large exocrine organ that packages the eggs into a cordon (Coggeshall, 1972); it is the first and largest exocrine organ contacted by the eggs. The second potential tissue of origin is the atrial gland (Fig. 1), a smaller exocrine organ that secretes into the oviduct (Arch *et al.*, 1980; Beard *et al.*, 1982; Painter *et al.*, 1985). The atrial gland is the last exocrine organ to contact the egg cordon, but unlike those of the albumen gland, the timing and general function of its secretion are not known.

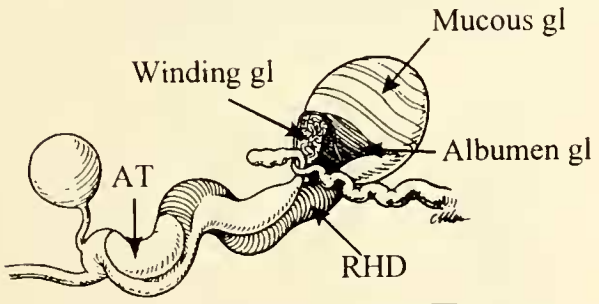

Figure 1. Schematic diagram of the reproductive tract of *Aplysia californica* rostral to the ovotestis (which would be at the far right). The labeled structures are exocrine organs that come in contact with the egg cordon during deposition. The albumen, mucous, and winding glands package the eggs into a cordon. The red hemiduct (RHD) is the functional oviduct that transports the egg cordon to the common genital aperture (which would be at the far left). The RHD secretes onto the cordon, but neither the secretory products nor their functions have been identified. The atrial gland (AT) secretes into the oviduct, but the timing of secretion is not known. T-maze experiments have identified the albumen and atrial glands as potential tissue sources of pheromonal attractants.

The current studies take advantage of two significant observations: (1) egg laying can be induced in *Aplysia* by injecting atrial gland extract into the hemocoel (Arch *et al.*, 1978); and (2) recently deposited egg cordons are a source of water-borne pheromonal attractants (Painter *et al.*, 1991). When atrial gland extract is used to induce egg laying, the number of animals laying eggs, the timing and synchronization of egg deposition, the general area where eggs are deposited, and (to some extent) the purity of the surrounding artificial seawater (ASW) can be controlled. In terms of "cordon-derived" pheromones, this means that it is possible to control the timing of pheromone secretion, the amount of pheromone secreted, and the relative amount of contaminants in the seawater.

Using this approach, we have isolated a peptide pheromonal attractant from eluates of recently deposited egg cordons, obtained a partial NH_2-terminal sequence, and used molecular techniques to identify the albumen gland as the organ of origin. The full-length peptide was isolated from the gland, and its complete 58 amino acid sequence was determined and found to match the sequence predicted from an albumen gland cDNA. *N*-linked glycosylation was demonstrated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and attractiveness was verified in the T-maze assay.

To the best of our knowledge, this is the first water-borne peptide pheromone to be characterized in molluscs, and indeed in invertebrates. The only other water-borne peptide pheromones that have been characterized to date include a small family of structurally related mating pheromones from the ciliated protozoan *Euplotes* (*e.g.*, Raffioni *et al.*, 1988, 1992; Stewart *et al.*, 1992); several small families of mating pheromones in different genera of yeast (*e.g.*, *Saccharomyces*, Stotzler *et al.*, 1976; *Rhodospiridium*, Kamiya *et al.*, 1978); and a female attractant from the cloacal gland of male red-bellied newts (*Cynops*; Kikuyama *et al.*, 1995).

Materials, Methods, and Results

Animals

Two species of *Aplysia* were used in these studies because each has a unique characteristic that simplifies specific aspects of the experiments. This approach was feasible because the pheromonal attractants associated with the aggregation do not appear to be species-specific; breeding aggregations sometimes contain more than one species of *Aplysia* (Kupfermann and Carew, 1974; Achituv and Susswein, 1985), and the associated egg masses sometimes contain egg cordons from more than one species

(Achituv and Susswein, 1985). Only sexually mature animals, as judged by the ability to lay eggs, were used.

Aplysia brasiliana (Rang) was selected as the experimental animal for T-maze bioassays because it has lower levels of chance attraction in the maze than *A. californica* (Painter *et al.*, 1991), and it can be collected in large numbers from the south Texas coast during the reproductive season. The animals (130–390 g) were housed in individual cages in one of five large aquaria containing recirculating ASW (Instant Ocean Marine Salt, Longhorn Pet Supply, Houston, Texas). Water temperature was maintained at 20° ± 2°C; the salinity ranged from 30 to 32 ppt. A 14:10 light:dark cycle was maintained, with the light period starting at 0600 hours. Animals were fed dried laver in the late afternoon (1600–1800), after experiments had been completed. Egg-laying activity was checked twice every day (0800–0900, 1600–1800), activity recorded, and egg cordons removed.

Specimens of *Aplysia californica* (Cooper) were obtained from Alacrity Marine Biological Services (Redondo Beach, California) and maintained as described above, except that the water temperature was 14° ± 2°C. This species was used as the source of egg cordons for elutions because it lays eggs less frequently than *A. brasiliana* (Fig. 2A, B), leaving a larger proportion of animals that can be induced to lay eggs on any day, and the volume of the egg mass is larger (Fig. 2C, D; also see Dudek *et al.*, 1979; Pinsker and Parsons, 1985). *A. californica* was also used as the source of albumen glands in later experiments.

Induction of egg laying, collection of egg cordons, and elution

Procedures. Egg laying was induced by injecting 0.1 ml of atrial gland extract (made as described in Painter *et al.*, 1991) through the foot into the hemocoel. All specimens (250–500 g) of *A. californica* that had not laid eggs during the preceding 24 h were injected, the number varying in each elution, and most began laying within 30–40 min. One hour following the injection, and at 30-min intervals thereafter, egg cordons were severed a short distance from the common genital aperture and removed from the cage.

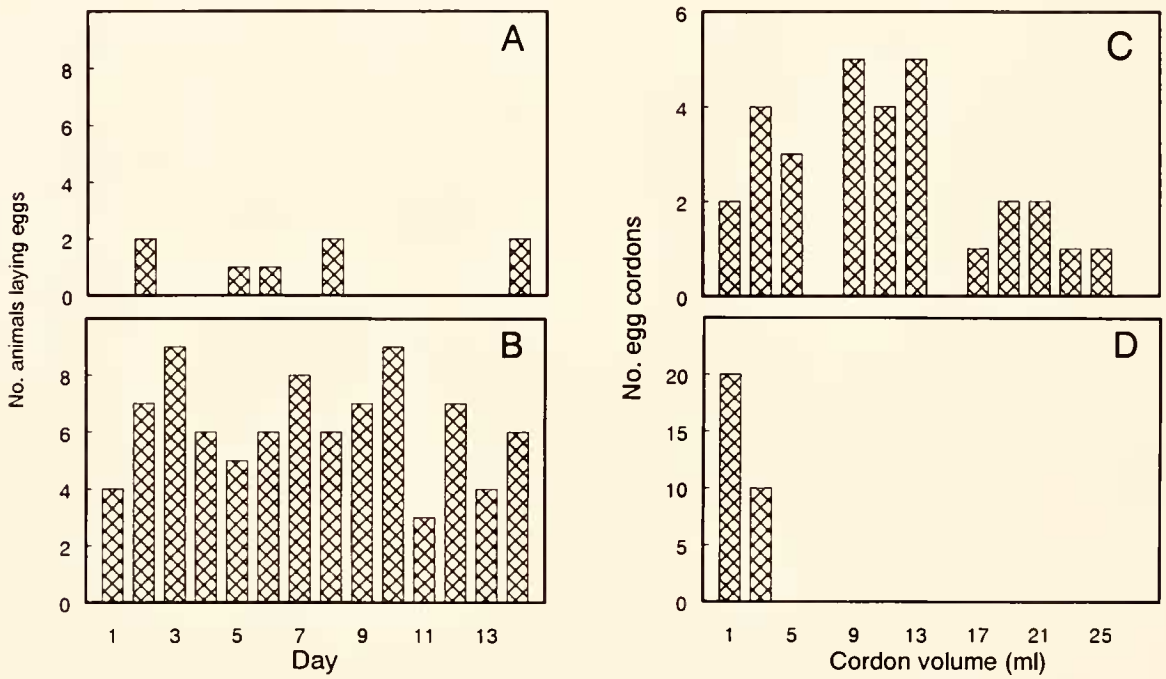

Figure 2. *Aplysia californica* lays eggs less frequently than *A. brasiliana*, and the egg cordons have a larger volume. A, B: The daily egg-laying history of *A. californica* (A) and *A. brasiliana* (B) over a 2-week period. Ten animals were present in each aquarium: all were known egg layers, and none was used in any other experiments during this time. Egg laying was checked every morning and egg cordons removed. Housing conditions differed only in water temperature. A total of 8 cordons were laid by *A. californica;* 87 cordons were laid by *A. brasiliana.* C, D: The volumes of 30 cordons laid by *A. californica* (C) and *A. brasiliana* (D); each cordon was laid by a different animal. Volume was measured by ASW displacement in a graduated cylinder. The mean volume of *A. californica* cordons was 11.1 ml, and the mean volume of *A. brasiliana* cordons was 1.5 ml.

When all of the cordons from a 30-min laying period had been collected, they were rinsed briefly in fresh ASW that had not previously contacted animals or egg cordons and then transferred to a beaker containing 200–300 ml of the fresh ASW for elution. When the volume of cordons was large (judged by eye before the rinse), two or three eluting beakers were used. Each beaker was covered and placed on a rotary shaker for 30 min. The water was at aquarium temperature when the eggs were added, and it increased by 1°–2°C during the elution period. After the elution, the cordons were carefully removed from each beaker and blotted to remove excess fluid. Cordon volume was then determined by ASW displacement in a graduated cylinder.

Several steps were taken to minimize the amount of material in the eluate that was not derived from the egg cordons. First, the ASW used for the rinses and elutions was made from isotonic salt solutions in glass-distilled water. This was necessitated by the large volumes of ASW used (small amounts of contaminants in the ASW became major components of the concentrated eluate). Second, the eluting beakers were thoroughly rinsed with this ASW and then used in two 30-min elutions comparable to those performed on the egg cordons. The first of these was discarded. The second was processed like the egg cordon eluates, and was fractionated by C18 reversed-phase (RP)-high performance liquid chromatography (HPLC). The resulting profile was used to identify water- and glassware-derived factors in the egg cordon eluates. Third, latex gloves were worn whenever egg cordons, cordon eluates, or tools that came in contact with either were handled.

Results. The number of *Aplysia* injected per elution ranged from 10 to 54, and egg deposition appeared to be normal. The injected animals continued to lay eggs for an extended period of time (4–8 h), and the mean volume of eggs laid (10.2 ml/animal; range: 9.1–15.2 ml/animal) was comparable to that of spontaneously laid egg cordons (11.1 ml/animal; Fig. 2C). The volume of cordons eluted during each period ranged from 1 to 125 ml, the variation reflecting the number of animals laying during the period and the amount of time each utilized for the activity. The volume provided a crude estimate of the amount of material and activity contained in each sample. Figure 3 shows the volume of eggs deposited during each interval of a 5-h deposition. The total volume of eggs collected for an elution ranged from 152 to 490 ml.

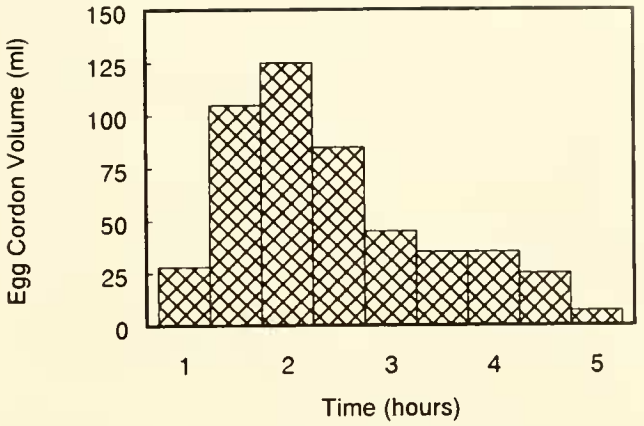

Figure 3. Time course of induced egg laying in *Aplysia californica*. In this experiment, 54 animals were injected with atrial gland extract at time zero, and most began laying eggs at about 30–40 min. Starting at 1 h post-injection, and at 30-min intervals thereafter, cordons were collected for elution. Following elution, the volume of the collected cordons was measured by ASW displacement in a graduated cylinder. The mean egg-cordon volume in this elution was 9.1 ml/animal; the mean for all elutions was 10.2 ml/animal.

Peptide purification

Procedures. After the cordons were removed, the eluates were filtered through a sterile disposable filter (0.45 μm cellulose acetate membrane; Fisher Scientific Products), acidified to 0.1% trifluoroacetic acid (TFA; VWR Scientific, reagent grade), and stored for a short time (usually less than 30 min) at 4°C in a sealed container until they could be further purified on a C18 Sep-Pak cartridge (Rainin Instrument Company). Two to three cartridges were used for each elution beaker. Each was pretreated with 3 ml of 60% acetonitrile (VWR Scientific, HPLC grade) containing 0.1% TFA, and rinsed with 5 ml of 0.1% TFA. The sample was passed through the cartridge once, and the cartridge was rinsed with 5 ml of 0.1% TFA. The peptides were then eluted with 3 ml of 60% acetonitrile containing 0.1% TFA, lyophilized, and stored at −20°C until use.

The lyophilized samples were resuspended in 1.5 ml of 0.1% heptafluorobutyric acid (HFBA; Pierce Chemical Company) and applied to a Vydac analytical C18 RP-HPLC column (0.46 × 25 cm). Both the guard and analytical columns had a 5-μm particle size. The column was eluted with a linear gradient of 0.1% HFBA and acetonitrile containing 0.1% HFBA. The column eluate was monitored at 215 nm and 1-min (1-ml) fractions collected.

Results. A representative RP-HPLC profile of an egg-cordon eluate is shown in Figure 4. Four major peaks (labeled 1–4 in the figure; referred to as C1–C4 in the text) were present in all elutions and had consistent retention times; the control RP-HPLC elution profiles showed that C1–C4 were not derived from the glassware or ASW. They were pooled based on absorbance, lyophilized, and stored at −20°C until they could be bioassayed for pheromonal attraction in the T-maze.

T-maze bioassays

Procedures. The T-maze, removable stimulus cages, and experimental protocol have been described previously

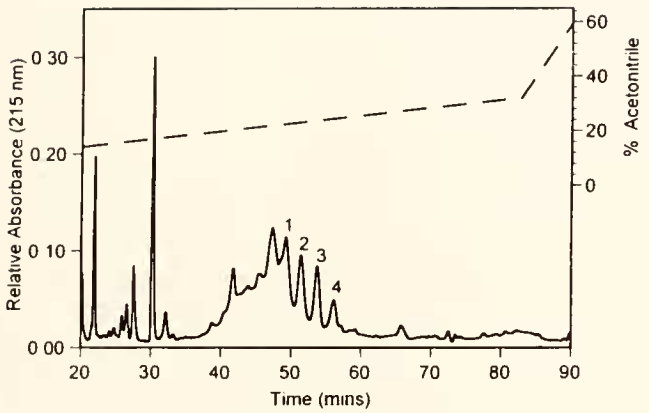

Figure 4. Representative fractionation of *Aplysia* egg-cordon eluates by C18 RP-HPLC. This eluate was from 35 ml of *A. californica* egg cordons; they were laid by 54 animals 180–210 min after injection with atrial gland extract. The fractions were eluted with a linear gradient (– – –) of 0.1% HFBA and acetonitrile containing 0.1% HFBA. Four peak fractions, C1–C4 (labeled 1–4), were pooled based on absorbance at 215 nm, and bioassayed for attraction in the T-maze.

(Painter *et al.*, 1991). Individual *A. brasiliana* for each assay were selected on the basis of four criteria: (1) the animal must be sexually mature but not have laid eggs during the preceding 24 h; (2) the animal must not have been used in a bioassay during the preceding 24 h; (3) test animals must not have been exposed previously to the fraction being tested; and (4) stimulus and test animals must be housed in the same aquarium. The potential attractants tested consisted of C1, C2, C3, or C4. The amount of material that could be recovered from 2 ml of egg cordons was tested; 2 ml is about 20% of the volume of egg cordons laid by one animal.

The test animal was placed at the base of the maze 5 min after the stimulus animal and potential attractant were placed in one of the stimulus cages. In most cases, the test animal moved directly to the top of the maze and exhibited one of two patterns of behaviors: (1) it stopped, moved its head from side to side, then either moved into one arm or returned to the base of the maze and remained there; or (2) it swam around in the maze, often visiting both cages before deciding where to stop. A response was considered to be positive if the test animal traveled to the stimulus within 20 min and maintained contact with the stimulus cage for 5 min, negative if the test animal traveled to the opposite arm and maintained contact with the cage for 5 min, and no choice if it did neither. Fifteen experiments were performed for each potential attractant, and the attractant was alternated between arms in consecutive experiments. Statistical significance was assessed by χ^2 analyses.

Results. To assess directional bias and chance levels of attraction in the maze, 15 experiments were performed in which the stimulus consisted of a nonlaying animal with nothing added to the surrounding ASW. Previous studies (Painter *et al.*, 1991) have shown that nonlaying *A. brasiliana* individuals are as attractive as empty stimulus cages. Two animals (13.3%) traveled to the right arm and remained, three (20%) traveled to the left arm and remained, and ten (66.7%) did neither. Of the five animals making a choice, only three went to the stimulus animal, one of which was in the right arm and two of which were in the left arm of the maze. These bioassays demonstrate that there was no directional bias in the maze and establish chance levels of attraction at 2–3 animals.

The positive control consisted of an extract of the atrial gland (equivalent to 0.5 gland) placed in the ASW adjacent to the stimulus animal. When the extract was present, a larger number of animals were attracted to the stimulus (11 animals; 73.3%) and fewer made negative (1 animal; 6.7%) and no-choice (3 animals; 20%) responses (Fig. 5). The change in response pattern was statistically significant [$\chi^2(2) = 7.85$; $0.01 < P < 0.025$].

A similar change in response pattern was observed when any of the fractions of egg-cordon eluate were placed in the ASW: more animals were attracted to the stimulus and fewer failed to make a choice (Fig. 5). The magnitude of the pattern changes differed among the fractions, with C1 producing the largest and most significant change: 13 of 15 animals (86.7%) were attracted to the stimulus, 1 animal (6.7%) chose the opposite arm, and 1 animal (6.7%) failed to make a choice. This change was statistically significant [$\chi^2(2) = 12.16$; $P < 0.005$]. Fewer animals were attracted by the other cordon fractions, and the resulting changes in response pattern were not statisti-

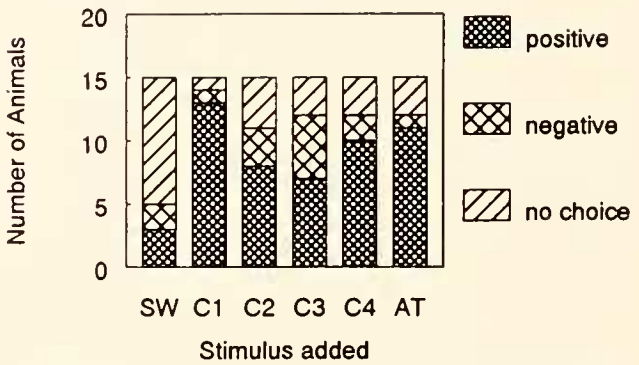

Figure 5. Fractions C1–C4 of egg-cordon eluate may contain pheromonal attractants. The number of *Aplysia brasiliana* attracted to a nonlaying conspecific (SW) was increased when any of the cordon fractions was placed in the adjacent seawater, and fewer animals failed to make a choice. The positive control (AT) is an atrial gland extract equivalent to 0.5 gland. This bar graph is based on 90 single-arm experiments, 15 per stimulus. In each experiment, animals chose between a stimulus in one arm and no stimulus in the other. The change in response pattern was statistically significant only for fraction C1 and the positive control.

cally significant [C2: $\chi^2(2) = 3.38$, $0.1 < P < 0.25$; C3: $\chi^2(2) = 4.51$, $0.1 < P < 0.25$; C4: $\chi^2(2) = 5.93$, $0.05 < P < 0.1$].

All four fractions also induced behaviors that might have resulted in male mating activity if the animals had had greater access to each other. These included eversion of the penis, attempting to enter the stimulus cage, pushing the oral veil through holes in the cage, and physically interacting with the stimulus animal. The number of times such behaviors occurred differed among the fractions, with C1 producing the largest number (Table I).

Differences in the magnitude of fraction activity could reflect differences in the amount of peptide recovered from the cordon eluate (as indicated by differences in peak area), the potency of the recovered peptides, the purity of the fractions, or some combination of the three. Because it was not easy to distinguish among these possibilities, all four fractions were subjected to biochemical analysis.

Compositional and microsequence analyses

Procedures. Reagents for these analyses were purchased from Perkin-Elmer/Applied Biosystems. Compositional analyses were performed using an Applied Biosystems 420H amino acid analyzer. For microsequence analyses, samples were applied to a Perkin Elmer/Applied Biosystems Procise 494/HT protein/peptide sequencer. The PTH derivatives of the amino acids obtained from the sequencer were identified and quantified by HPLC (Nagle *et al.*, 1986). Some samples were reduced and alkylated prior to application.

Results. The compositions of the four fractions are shown in Table II. Partial sequences, consisting of at least 24 residues for each peptide fraction, were obtained and are summarized in Table III. The sequences are identical except for length. Each has multiple cysteine residues, and one potential *N*-linked glycosylation site (Asn^8). The number of picomoles of Asn recovered at position 8 is about the same as was recovered for Gly at position 7 and for Ile at position 9, suggesting that the peptides were not glycosylated.

Table I

Potential male sexual behavior in maze assay

Stimulus	Behaviors				
	Evert penis	Enter cage	Animals interact	Stimulus animal moves to stimulus	Total animals
Non-egg-laying *Aplysia*	0	0	0	0	0
C1	3	3	1	2	9
C2	0	0	1	0	1
C3	0	0	3	2	5
C4	0	1	4	0	5
Atrial gland extract	0	1	0	0	1

Values represent number of animals exhibiting each behavior.

Table II

Amino acid composition (residues/mol peptide) of attractin-related fractions of egg-cordon eluate and of attractin from albumen gland

Amino acid	Fractions[a]				Attractin[b]
	C1	C2	C3	C4	
Cysteine	ND	ND	ND	ND	6
Aspartic acid	10.4	10.1	11.0	8.8	9
Threonine	5.3	6.0	6.2	6.3	5
Serine	2.9	2.5	1.9	1.9	4
Glutamic acid	11.4	10.0	6.8	7.4	13
Proline	1.4	1.6	1.8	2.2	1
Glycine	3.3	3.2	2.3	4.1	4
Alanine	2.8	2.7	1.9	2.5	3
Valine	1.3	1.5	1.8	2.1	1
Methionine	1.2	0.8	0.6	0.4	2
Isoleucine	4.6	4.6	4.8	4.8	4
Leucine	1.2	1.3	1.5	1.9	1
Tyrosine	0.1	0	0	0.2	0
Phenylalanine	1.0	1.0	0.8	1.0	1
Lysine	1.7	1.2	0.5	0.5	2
Arginine	0.8	0.6	0.2	0.4	1
Tryptophan	ND	ND	ND	ND	0
Histidine	0.9	0.6	0.2	0.5	1

[a] Residues determined by compositional analysis following hydrolysis. ND = not determined.

[b] Residues determined by sequence analysis following reduction and alkylation with 4-vinylpyridine.

Molecular studies to identify the organ of origin

Because of the difficulties inherent in generating egg-cordon eluates for analysis and the probability that degradation is occuring during the process, we next focused on identifying the pheromonal organ of origin; a molecular approach was used. Degenerate oligonucleotide primers corresponding to the NH_2- and COOH-terminal regions of the longest characterized peptide (C1) were used to generate an albumen-gland polymerase chain reaction (PCR) product. The PCR product was cloned and sequenced; it encoded the 46-residue peptide sequence. The corresponding cDNA was isolated from an albumen gland cDNA library and the entire 76-residue precursor sequence predicted (Fan *et al.*, 1997). The predicted sequence of the full-length 58-residue peptide ("attractin") is shown in Table IV.

The attractin cDNA was expressed in the baculovirus system, and lysates were fractionated by RP-HPLC under the same gradient conditions as the egg-cordon eluates

Table III

Sequence and quantity (in picomoles) of amino acid residues, determined by automated sequence analysis, of attractin-related fractions of egg-cordon eluate and of attractin from albumin gland

Edman cycle	C1 (U)	C2 (R/A)	C3 (U)	C3 (R/A)	C4 (U)	C4 (R/A)	Attractin 1A (R/A)	Attractin 1B (R/A)	Attractin 48–58 (D)
0*	— (1250)	— (990)	— (250)	— (205)	— (380)	— (100)	— (232)	— (320)	— (170)
1	Asp (648)	Asp (39)	Asp (20)	Asp (70)	Asp (31)	Asp (33)	Asp (71)	Asp (80)	Ser (41)
2	Gln (1028)	Gln (37)	Gln (35)	Gln (86)	Gln (34)	Gln (22)	Gln (73)	Gln (210)	Ala (119)
3	Asn (787)	Asn (38)	Asn (20)	Asn (65)	Asn (27)	Asn (23)	Asn (113)	Asn (145)	Ala (123)
4	ND —	Cys (33)	ND —	Cys (98)	ND —	Cys (15)	Cys (260)	Cys (363)	Gly (97)
5	Asp (563)	Asp (25)	Asp (15)	Asp (38)	Asp (21)	Asp (20)	Asp (82)	Asp (104)	Ser (40)
6	Ile (767)	Ile (43)	Ile (18)	Ile (88)	Ile (18)	Ile (33)	Ile (198)	Ile (280)	Thr (55)
7	Gly (592)	Gly (26)	Gly (7)	Gly (47)	Gly (15)	Gly (15)	Gly (150)	Gly (217)	Thr (57)
8	Asn (538)	Asn (36)	Asn (11)	Asn (54)	Asn (16)	Asn (20)	Asn (14)	ND —	Leu (77)
9	Ile (674)	Ile (34)	Ile (6)	Ile (71)	Ile (17)	Ile (21)	Ile (194)	Ile (274)	Gly (50)
10	Thr (460)	Thr (17)	Thr (5)	Thr (40)	Thr (9)	Thr (12)	Thr (85)	Thr (141)	Pro (37)
11	Ser (206)	Ser (9)	Ser (3)	Ser (19)	Ser (4)	Ser (10)	Ser (47)	Ser (69)	Gln (11)
12	Gln (422)	Gln (18)	Gln (7)	Gln (44)	Gln (11)	Gln (10)	Gln (95)	Gln (147)	
13	ND —	Cys (18)	ND —	Cys (42)	ND —	Cys (12)	Cys (148)	Cys (237)	
14	Gln (390)	Gln (17)	Gln (6)	Gln (32)	Gln (6)	Gln (8)	Gln (103)	Gln (146)	
15	Met (319)	Met (12)	Met (4)	Met (42)	Met (5)	Met (6)	Met (115)	Met (196)	
16	Gln (312)	Gln (15)	Gln (4)	ND —	Gln (5)	Gln (11)	Gln (83)	Gln (126)	
17	His (91	His (4)	His (1)	His (8)	His (1)	His (5)	His (35)	His (65)	
18	Lys (116)	Lys (3)	Lys (1)	Lys (6)	Lys (1)	ND —	Lys (58)	Lys (107)	
19	Asn (236)	Asn (11)	Asn (4)	Asn (27)	Asn (2)	Asn (10)	Asn (29)	Asn (58)	
20	ND —	Cys (7)	ND —	Cys (30)	ND —	Cys (8)	Cys (53)	Cys (113)	
21	Glu (124)	Glu (8)	Glu (2)	Glu (15)	Glu (2)	ND —	Glu (33)	Glu (77)	
22	Asp (95)	Asp (8)	Asp (1)	Asp (27)	Asp (2)	Asp (2)	Asp (15)	Asp (30)	
23	Ala (113)	Ala (6)	Ala (1)	Ala (16)	Ala (1)	Ala (3)	Ala (28)	Ala (54)	
24	Asn (110)	Asn (10)	Asn (1)	Asn (9)	Asn (1)	ND —	Asn (32)	Asn (31)	
25	Gly (85)	Gly (3)	Gly (2)				Gly (18)	Gly (40)	
26	ND —	Cys (2)	ND —				Cys (16)	Cys (50)	
27	Asp (36)	ND —	Asp (38)				Asp (11)	Asp (18)	
28	Thr (35)	Thr (1)	ND —				Thr (7.8)	Thr (20)	
29	Ile (42)	Ile (2)	Ile (11)				Ile (11)	Ile (33)	
30	Ile (81)	Ile (2)					Ile (29)	Ile (64)	
31	Glu (24)	Glu (3)					Glu (4.8)	Glu (20)	
32	Glu (37)						Glu (15)	Glu (35)	
33	ND —						Cys (6.8)	Cys (22)	
34	Lys (10)						Lys (5.8)	Lys (17)	
35	Thr (13)						Thr (0.4)	Thr (7.7)	
36	Ser (11)						Ser (0.9)	Ser (4.6)	
37	Met (11)						Met (3.5)	Met (14)	
38	Val (12)						Val (3.2)	Val (12)	
39	Glu (7)						Glu (0.4)	Glu (4.7)	
40	Arg (8)						Arg (3.6)	Arg (11)	
41	ND —						Cys (1.7)	Cys (10)	
42	Gln (3)						Gln (2.1)	Gln (6.4)	
43	Asn (4)						Asn (2.5)	Asn (4.6)	
44	Gln (8)						Gln (4.3)	Gln (13)	
45	Glu (1)						Glu (0.4)	Glu (5.0)	
46	Phe (4)						Phe (1.1)	Phe (6.6)	
47							ND —	Glu (4.3)	
48							Ser (0.4)	Ser (2.1)	
49							Ala (1.2)	Ala (4.4)	
50							Ala (1.2)	Ala (8.4)	
51							Gly (0.7)	Gly (1.3)	
52							ND —	Ser (0.1)	
53							ND —	Thr (1.5)	
54							ND —	Thr (1.5)	
55							Leu (1.4)	Leu (1.2)	
56								ND —	
57								Pro (2.4)	

Samples of egg-cordon eluate (C1–C4) were either untreated before sequence analysis (U) or were reduced and alkylated with 4-vinylpyridine (R/A). Samples of attractin from albumin gland were either reduced and alkylated with 4-vinylpyridine (R/A) or were digested with Endo Glu-C (D) so that the COOH-terminal fragment (residues 48–58) could be identified and sequenced. ND = not determined.

* Initial amount of peptide (picomoles) applied to sequencer.

Table IV

Amino acid sequences of Aplysia californica *peptides from attractin-related fractions of egg-cordon eluate and from attractin*

	10	20	30	40	50
Att	DQNCDIGNITSQCQMQHKNCEDANGCDTIIEECKTSMVERCQNQEFESAAGSTTLGPQ				
C1	DQN-DIGNITSQ-QMQHKN-EDANG-DTIIEE-KTSMVER-QNQEF				
C2	DQNCDIGNITSQCQMQHKNCEDANGC-TIIE				
C3	DQNCDIGNITSQCQMQHKNCEDANG-D-I				
C4	DQNCDIGNITSQCQMQHKNCEDAN				

Attractin (Att) is glycosylated at Asn^8. Fractions C2, C3, and C4 were reduced and alkylated before analysis; C1 was not.

(data not shown). The peak fraction containing full-length attractin was identified by sequence analysis, and the retention time was used in subsequent studies to identify full-length attractin in extracts of the albumen gland.

Isolation and characterization of albumen gland attractin

Procedures. Albumen glands were removed from sexually mature individuals of *A. californica*, extracted at 4°C in 0.1% HFBA using a Polytron homogenizer, and sonicated. The extract was centrifuged for 20 min at 48,000 $\times g$ (4°C) and the supernatant Sep-Pak purified. The range of acetonitrile concentrations was narrowed from 0%–60% to 10%–50% in the Sep-Pak procedure to minimize the large peaks eluting very early and very late in the RP-HPLC gradient. The purified sample was fractionated by RP-HPLC, using the same gradient conditions as the egg-cordon eluates. The peak of interest was identified from the retention time of recombinant attractin.

To verify the 46-residue NH_2-terminal sequence, aliquots of the peak fraction were reduced with 2-mercaptoethanol, alkylated with 4-vinylpyridine, and purified by RP-HPLC with TFA as counterion. The peak of interest was identified by compositional analysis and subjected to microsequence analysis. To obtain the COOH-terminal sequence, samples were digested with sequencing grade endoproteinase Glu-C (Endo Glu-C; Boehringer-Mannheim), and the resulting fragments were purified by RP-HPLC with TFA as counterion. The COOH-terminal peptide was detected by compositional analysis and subjected to microsequence analysis.

Results. A representative RP-HPLC elution profile from extracts of albumen glands from *A. californica* is shown in Figure 6A. Fraction 1 coeluted with recombinant attractin. It was reduced, alkylated, and then purified by RP-HPLC with TFA as counterion (Fig. 6B). Fractions 1A and 1B were subjected to microsequence analysis for 55 and 57 residues, respectively; the sequences were identical (Table III), but incomplete. To determine the COOH-terminal sequence, Fraction 1 (Fig. 6A) was digested with Endo Glu-C and the resulting fragments purified by RP-HPLC (Fig. 6C). The COOH-terminal fragment was identified by compositional analysis and sequenced. The sequence obtained (Table III) was identical to that predicted for residues 48–58 of attractin by nucleotide sequence analysis of the attractin cDNA (Fan *et al.*, 1997).

No major albumen gland peaks coeluted with cordon eluate fractions C1–C4, suggesting that these fractions were generated by extracellular degradation of the full-length peptide. In addition, the recovery of Asn at position 8 was reduced by several hundredfold relative to adjacent amino acids in the sequence of albumen gland attractin, but not in fractions C1–C4. This suggests that the peptide is glycosylated in the cell and that extracellular degradation removes the carbohydrate.

MALDI-TOF mass spectrometry

Procedures. N-linked glycosylation was investigated using MALDI-TOF MS of both the full-length native attractin and the reduced and alkylated attractin. The matrix employed (2,5-dihydroxybenzoic acid; DHB; ICN Pharmaceuticals, Costa Mesa, California) readily produces stable protonated molecular ions of glycopeptides (Harmon *et al.*, 1996). Samples were prepared by mixing a 0.5-μl aliquot of $\sim$7 μM attractin with 0.5 μl of 65 mM aqueous DHB on a metallic sample probe. The probe was dried at ambient temperature and then inserted into the mass spectrometer.

MALDI-TOF MS experiments were performed with a Voyager Elite biospectrometry research station with delayed ion extraction (PerSeptive Biosystems, Framingham, Massachusetts). A pulsed nitrogen laser (337 nm) served as the desorption/ionization source, and positively charged ions were detected in the reflectron mode using a 200-ns delay prior to acceleration at 20 kV. The laser beam was focused at the sample surface with enough energy to reach the ionization threshold. Each mass spectrum shown is the average of $\sim$100 laser pulses, with mass calibration performed externally using the known $[M + H]^+$ and $[M + 2H]^{2+}$ peaks from MALDI-TOF MS of bovine insulin (Sigma Chemical Company, St. Louis, Missouri).

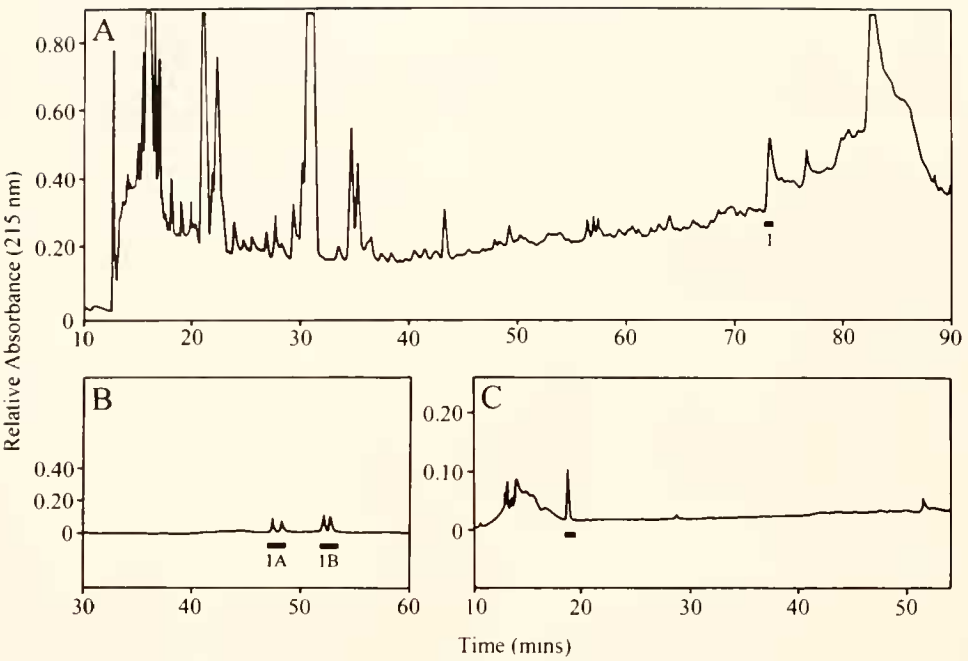

Figure 6. Representative fractionation of albumen gland extracts by RP-HPLC. (A) The extract was eluted with a linear gradient of 0.1% HFBA and acetonitrile containing 0.1% HFBA. The peak fraction 1 was pooled based on absorbance at 215 nm. (B) Fraction 1 (A) was reduced and alkylated with 4-vinylpyridine, and fractionated with a linear gradient of 0.1% TFA and acetonitrile containing 0.1% TFA. Peak fractions 1A and 1B were pooled based on absorbance and sequenced. (C) An aliquot of fraction 1 (A) was digested with Endo Glu-C and the resulting fragments fractionated with a linear gradient of 0.1% TFA and acetonitrile containing 0.1% TFA. The peak fraction indicated by the solid bar was sequenced and corresponded to attractin residues 48–58.

Results. As shown in Figure 7, MALDI-TOF MS of attractin detected singly and doubly charged components. Examining the singly charged group, inset 7A shows three major peaks at m/z 8059, 8075, and 8091. To investigate these three peaks, additional mass spectra were acquired from attractin that had been in solution at room temperature for over 3 h. This produced a similar mass spectra with differences in intensities of the three major peaks (shown in inset 7B). Several satellite peaks are observed spaced 162.1 ± 0.3 apart. Furthermore, mass spectra acquired from reduced and alkylated attractin (data not shown) contained one major peak at m/z 8696, along with two series of small peaks spaced 162.1 ± 2.3.

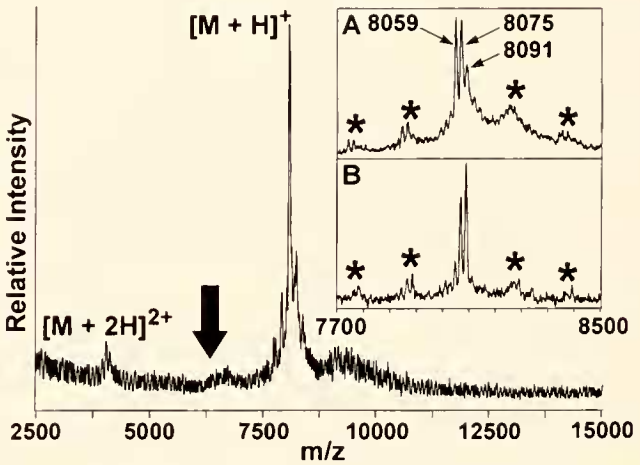

Figure 7. MALDI-TOF mass spectrometry reveals that full-length attractin is glycosylated. Mass spectra contain singly and doubly charged peaks corresponding to M_r 8059 Da for the major form. The satellite peaks (indicated by asterisks in the insets) are spaced by single hexose units. The spectrum in inset A was obtained from a freshly prepared solution, whereas the attractin in inset B had been in solution for over 3 h. Peak intensities at m/z 8075 (attractin + 16) and 8091 (attractin + 32) vary over time, confirming the presence of two readily oxidized Met residues.

T-maze bioassay

Procedures. To verify that attractin is an attractive component of fractions C1–C4 of the egg-cordon eluate, the full-length peptide was purified from the albumen gland as described above, aliquoted into 10-pmol samples, and bioassayed in the T-maze.

Results. The results are summarized in Figure 8. In the negative control (nonlaying conspecific with nothing placed in the adjacent ASW), three animals (20%) trav-

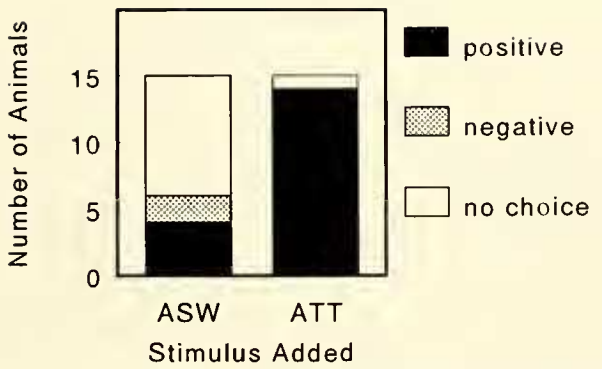

Figure 8. Attractin is a water-borne pheromonal attractant. The number of *Aplysia brasiliana* attracted to a nonlaying conspecific (ASW) was increased when full-length attractin (10 pmol) was placed in the adjacent seawater; fewer animals went to the opposite arm and fewer failed to make a choice. This bar graph is based on 30 single-arm experiments, 15 per stimulus. In each experiment, animals chose between a stimulus in one arm and no stimulus in the other. The change in response pattern is statistically significant ($\chi^2(2) = 8.04$; $0.01 < P < 0.025$).

eled to the right arm and remained, three (20%) traveled to the left arm and remained, and nine (60%) did neither. Of the six animals making a choice, only four went to the stimulus animal, two of which were in the right arm and two of which were in the left arm of the maze. These bioassays verify that there is no directional bias in the maze and establish chance levels of attraction at 4 animals.

The response pattern changed when 10 pmol of attractin was placed in the seawater adjacent to the stimulus animal (Fig. 5): 14 of 15 animals (93.3%) were attracted to the stimulus, and one (6.7%) failed to make a choice. The change was statistically significant [$\chi^2(2) = 8.04$, $0.01 < P < 0.025$]. Attractin also induced behaviors related to male approach and mating: four test animals attempted to enter the stimulus cage, one of which succeeded and mated as a male; three put their oral veil through holes in the cage; four (not including the one mating as a male) made physical contact with the stimulus animal and interacted with it. The mating animals reversed roles after about 20 min, as they normally would (Leonard and Lukowiak, 1987).

Discussion

We have isolated a water-borne pheromonal attractant for *Aplysia* from eluates of recently deposited egg cordons, and have obtained a partial 46 amino acid sequence. Degenerate oligonucleotide primers corresponding to the NH_2- and COOH-terminal regions of this peptide were used to generate an albumen gland RT-PCR product encoding the 46-residue sequence. The corresponding cDNA was isolated from an albumen gland cDNA library and the entire 76 amino acid precursor sequence predicted, including the 58-residue peptide "attractin" (Fan *et al.*, 1997).

Attractin cDNA was expressed in the baculovirus system and in lysates fractionated by C18 RP-HPLC to determine the retention time of the full-length recombinant peptide (data not shown). This information was used to identify the attractin peak in extracts of the albumen gland and to verify that the full-length peptide was not present in the egg-cordon eluates. Northern analyses showed that the albumen gland was the only source of attractin in the female reproductive tract (Fan *et al.*, 1997), suggesting that extracellular degradation occurred.

The peak that coeluted with recombinant attractin was repurified by RP-HPLC using a different counterion and bioassayed in the T-maze. Like C1, the repurified peptide attracted a larger number of animals to the nonlaying conspecific (14 rather than 4) and produced a variety of behaviors that might have resulted in male behavior if the animals had had more access to each other. The change in response pattern was statistically significant, verifying that the peptide is attractive. The amount of peptide tested (10 pmol in 6 l) is consistent with pheromonal activity and is about the same as was tested for fraction C1 (estimate based on peak area). We are currently examining the potential effect of attractin on male mating behavior.

The isolated peptide was 58 amino acids in length, and the sequence matched that predicted from the albumen gland cDNA (Fan *et al.*, 1997). It contained a potential *N*-linked glycosylation site (Asn^8) and six cysteine residues, characteristics which could contribute to the hydrophilicity and stability of the peptide. It had less than 18% sequence identity with other sequences in the Non-Redundant Genbank CDS Database, demonstrating that it is a novel peptide.

To confirm glycosylation, we characterized the peptide by MALDI-TOF MS. No ions were detected at m/z 6351 (black arrow in Fig. 7); this is the protonated M_r corresponding to the full 58-residue peptide. Instead, we observed that attractin is indeed a glycopeptide having a major form with M_r 8058 Da, an increase of 1708 Da from the predicted mass, yielding 21 wt. % carbohydrate. Mass spectra indicated the presence of not one, but a series of five peaks, separated by 162.1 Da, the M_r of a single hexose unit.

This distribution was not unexpected, as glycoforms generally exist as a set of variants exhibiting microheterogeneity with respect to the oligosaccharide composition. The branched $(mannose)_3(GlcNAc)_2$ core of *N*-linked oligosaccharides accounts for 893 Da, while the remaining 815 Da can be assigned to an additional five hexose units. Attractin with $(hexose)_5(mannose)_3(GlcNAc)_2$ attached is

within 0.06% of the observed mass, well within the mass calibration of the instrument.

The major peaks detected at m/z 8057 and 8091 are consistent with attractin containing one and two oxidized Met residues, respectively. Non-physiological oxidation of Met to Met-sulfoxide readily occurs during sample handling, and MALDI-TOF MS can be used to monitor this occurrence over time (Zhang and Capriololi, 1996). As shown in the insets of Figure 7, the intensity of the peaks corresponding to the presumed oxidized forms increases over time, consistent with oxidation. Moreover, because two oxidized forms are detected, the presence of two Met residues within attractin is confirmed.

Mass spectra were also obtained from a sample of full-length attractin that had been reduced and alkylated with 4-vinylpyridine. Again, one major component was present in addition to smaller peaks separated by ±hexose units. Because the mass change associated with the reduction and alkylation of Cys is 105 Da per residue, we confirm the presence of six Cys residues, with the calculated (8689 Da) and determined (8695 Da) M_r within 0.06%. The six Cys residues may form three intramolecular disulfide bonds, providing a compact conformation for the peptide and greater stability. Unfortunately, the mass resolution of the instrument is insufficient to confirm this structure. The question is still under investigation.

Assuming that intramolecular disulfide bonds are formed, the family of mating pheromones in the ciliate protozoan *Euplotes* may serve as a good model for *Aplysia* attractin. In the *Euplotes* family, the pheromones are slightly shorter than attractin (38–40 amino acids), but like attractin, each peptide has six cysteine residues. The cysteines form intramolecular disulfide bonds in the *Euplotes* pheromones, forming three stable loops that differ in both size and charge from one pheromone to another within the family. The differences are thought to confer mating-type specificity, whereas the conserved sequences contribute to the conserved structure and preserve function (Raffioni *et al.*, 1988, 1989, 1992).

To examine the possibility that a similar system exists within attractin, we are now isolating and characterizing attractin-related peptides from other *Aplysia* species and examining their behavioral effects. We are comparing the peptide from sets of species that have (1) overlapping ranges and are sometimes seen in (or associated with) the same aggregations; (2) overlapping ranges and are not known to associate with the same aggregations; and (3) ranges that do not overlap. We have isolated an attractin-related peptide from the *A. brasiliana* albumen gland and have obtained a partial 50-residue N-terminal sequence; it is identical to the *A. californica* sequence at 48 positions. This peptide probably serves a pheromonal function in *A. brasiliana* and accounts, at least in part, for the observed attraction in the T-maze.

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